INVESTIGATION OF BACTERIAL PROFILE PRESENT IN PRIMARY AND SECONDARY ENDODONTIC INFECTIONS BY NESTED AND MULTIPLEX POLYMERASE CHAIN REACTION – AN INVIVO STUDY

Dissertation Submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY

In Partial Fulfillment for the Degree of

MASTER OF DENTAL SURGERY

BRANCH IV

CONSERVATIVE DENTISTRY AND ENDODONTICS

APRIL 2013

CERTIFICATE

This is to certify that this dissertation titled **"INVESTIGATION OF BACTERIAL PROFILE PRESENT IN PRIMARY AND SECONDARY ENDODONTIC INFECTIONS BY NESTED AND MULTIPLEX POLYMERASE CHAIN REACTION – AN IN VIVO STUDY"** is a bonafide record work done by **Dr. MURALI .S** under our guidance during his postgraduate study period between 2010 - 2013.

This dissertation is submitted to **THE TAMILNADU Dr. M.G.R.MEDICAL UNIVERSITY**, in partial fulfillment for the degree of **MASTER OF DENTAL SURGERY – CONSERVATIVE DENTISTRY AND ENDODONTICS, BRANCH IV**. It has not been submitted (partial or full) for the award of any other degree or diploma.

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ACKNOWLEDGEMENT

I take this opportunity to express my heartfelt gratitude to my post graduate teacher, mentor and guide ***Dr. Anil Kumar M.D.S., Professor,*** *Department of Conservative Dentistry & Endodontics, Ragas Dental College, for his untiring perseverance in motivating and supporting me throughout my postgraduate curriculum, for his friendly encouragement and meticulous care in correcting my mistakes. I thank him for all his guidance without which this dissertation would not have come true.*

Words seem less to express my deep sense of gratitude to my professor and mentor, ***Dr. R. Indira M.D.S., Professor*** *and* ***HOD*** *Department of Conservative Dentistry & Endodontics, Ragas Dental College for her invaluable guidance, unflinching support, keen surveillance, tireless pursuit for perfection and encouragement throughout my post graduate curriculum.*

I sincerely thank ***Dr. S. Ramachandran M.D.S., Professor*** *and* ***Principal****, Department of Conservative Dentistry & Endodontics, Ragas Dental College, who immensely supported me during my entire postgraduate curriculum.*

I earnestly thank ***Dr. C.S. Karumaran M.D.S., Professor, Dr. M. Rajasekaran M.D.S., Professor and Dr. Revathi Miglani M.D.S., D.N.B., Professor,*** *Department of Conservative Dentistry & Endodontics, Ragas Dental College, who always helped me with their valuable advice and supported me whenever I was in need.*

I take this opportunity to sincerely thank ***Mrs. Mahalakshmi, Microbiologist, Balaji Science and Research Institute, Chennai*** *for assisting me with the microbiological investigations. She was extremely helpful, patient and interested throughout the course of the study.*

I would like to solemnly thank ***Dr. Veni Ashok, M.D.S., Reader,*** *Department of Conservative Dentistry and Endodontics for all the help during my study period.*

I would also like to thank ***Dr. G. Shankar Narayan***, ***Dr. S.M. Venkatesan, Dr. Janani, Senior Lecturers,*** *Department of Conservative Dentistry and Endodontics for answering and solving the countless queries that I put to them during the course of my post graduation.*

I will forever remain grateful to my ***batch mates*** *who always inspired me, made me feel at home and made the three years of post graduation a memorable and unforgettable journey.*

*I take this opportunity to thank all my **postgraduate colleagues**, **juniors** and **friends** for their help and good wishes. I earnestly thank the **support staff** and **nurses** of the Department of Conservative Dentistry and Endodontics, Ragas Dental College for helping me during the course of my dissertation.*

***I have grown up with the wisdom of your words, cheerfulness of your laughter, strength of your mind and the warmth of your love.** I have reached this far in life only because of the countless sacrifices made by my father **Mr. R. Sivakumar** and my mother **Mrs. Usha Sivakumar.** I shall always be indebted to them for making me what I am today.*

*Above all else, I am grateful to the **"Almighty"**, who has blessed me with such wonderful people and has given me the opportunity to seek knowledge.*

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Introduction

INTRODUCTION

The success of endodontic treatment depends on various factors such as diagnosis, thorough cleaning and shaping, disinfection and three dimensional obturation of the pulpal space followed by a coronal seal. Elimination of microorganisms in the infected root canals directly influences the outcome of endodontic treatment. The classical study by Kakehashi et al proves that the presence of microbiota is the major deterrent in endodontic infections.[14] Microorganisms found in endodontic infections enter root canal via caries process, dentinal tubules, traumatic exposures, periodontal membrane and through blood stream (anachoresis).[2]

These microorganisms are capable of adhering, colonizing, surviving, propagating and at the same time can also evade host defence mechanism causing various pulpal and periapical pathoses. Thus preventing the microorganisms from infecting and re-infecting the root canal and/or periradicular tissues becomes the rationale of endodontic treatment.

Microorganisms inside the root canal can present in two forms. As Planktonic organisms, which are free floating bacteria and as

Biofilms, which are dense aggregates of microbes adhering to canal walls forming bacterial condensation.[22]

Root canal infections are classified as primary endodontic infection and secondary endodontic infection. Primary infection of root canal is the result of colonization of microorganisms in a necrotic pulp tissue leading to dysfunction of the pulp. Whereas, secondary infection in the root canals occurs due to the failure of endodontic treatment and are produced by microorganisms resistant to chemico-mechanical procedures or as a result of bacterial invasion through improper coronal restoration.

Studies have showed that the microbiota associated with the primary root canal infections differs from that of secondary root canal infections.[35-41] This is due to the fact that, there is change in the root canal environment such as type and availability of nutrients, oxygen tension and bacterial interactions all of which influences the specificity of root canal flora.[22] This favours the predominance of obligate anaerobes in primary endodontic infections and facultative anaerobes in secondary endodontic infections.[12,23,24]

Although studies in western countries tabulate the predominant microorganism present in root canal infections, literature on microorganisms isolated from Indian population is very sparse. Findings from the microbiological studies conducted at different parts of the globe has confirmed that a given species which is very prevalent in root canals of patients from some geographic region is not necessarily found in similar figures or even detected in samples from other geographic location.[38] Probably this possibility may exist because of the different composition of the oral microflora. Several studies have suggested that genetic and environmental factors may influence the composition of oral microbiota.[39]

The oral ecology can be subjective to following variation - microbial flora variation with geographic variation, the food habits that are followed, oral hygiene practices that are practiced, the environment and culture in which the people live and the treatment protocols that are being followed.[3,39]

Thus, identification of microorganisms in the root canal flora pertaining to Indian population would assist in determining effective antimicrobial therapies. This will enable us to tailor the treatment

protocol to favour the eradication of these microbes from the root canal space.

Traditionally, identification of root canal isolates were performed using standard cultural techniques but it is a known fact that only 50% of the bacteria in the oral cavity are cultivable. When identification of microorganisms in the root canal is considered, obtaining a representative sample is not often an easy task because of the physical constraints of the root canal system. This difficulty is far more pronounced in patients being retreated in whom the accessible microorganisms in the root canal can be low and a number of microbial cells can also be lost while attempting the procedures to remove the root canal filling.[36]

As a consequence, the number of cells sampled can fall short of the detection rate of the identification method and the prevalence of a given species can be under estimated. So, this demands a technique that can improve the sensitivity of microbial detection and thereby enable the identification of microorganisms with greater precision. In this regard, use of advanced molecular techniques, especially polymerase chain reaction based analysis of microorganisms has been proven to be beneficial. PCR assays are very sensitive and enable the reliable identification of microbial species or strains that are difficult or even impossible to culture.[36]

Aim:

The purpose of this present study was to, investigate the occurrence of microbial taxa in endodontic infections by means of sensitive microbial diagnostic tool: The PCR.

Objectives:

The objectives of this study was

1) To isolate and identify the microorganisms present in primary endodontic infections.
2) To isolate and identify the microorganisms present in secondary endodontic infections.

Hypothesis

The hypothesis tested was that there exists a difference in the microbiota in the root canal system according to different geographic locations.

Review of literature

REVIEW OF LITERATURE

Molander. A et al (1998)[17] examined the microbiological status of 100 root filled teeth with radiographically verified to be apical periodontitis. Facultative anaerobic species predominated among these isolates. Enterococci were the most frequently isolated genera, showing heavy or very heavy growth in 78% of cases and concluded that microflora of the obturated canal differs from that found normally in the untreated necrotic pulp, quantitavely as well as qualitatively.

Peciuliene et al (2000)[21] investigated the occurrence of *Enterococcus faecalis* in root canals of previously root filled teeth with apical periodontitis requiring retreatment in Lithuanian patients and found that *E . faecalis* was present in 14 out 20 teeth that showed positive cultures usually in pure culture form or a major component of the flora and concluded that ecological conditions present in the incompletely filled root canal are important for the presence of *E.faecalis* in such teeth.

H. J. Rolph et al (2001)[29] demonstrated that molecular technique can detect the presence of bacteria in endodontic infections

where culture techniques yield a negative result and can be used to identify a wider range of endodontic infection related bacteria including the presence of previously unidentified or uncultured bacteria.

Cheung GSP et al (2001)[5] investigated the composition of microflora in endodontically treated teeth associated with asymptomatic periapical lesions in southern Chinese patient. The number of bacterial genera recovered ranged between 0 – 6, with facultative gram positive cocci being the most prevalent group of bacteria isolated. Facultative anaerobic bacteria were present in all, whereas strict anaerobic bacteria were found in 3 out of 12 teeth with positive growth. The size of the periapical rarefaction did not show any relationship with the quantity of the microorganism recovered.

Ashraf F.Fouad et al (2002)[10] used primers to target the 16 S rRNA gened to identify 10 putative bacterial pathogens in root canals with necrotic pulp, and out of 24 samples, bacteria were found in 22 samples and showed that *Streptococcus* species are significantly associated with pre operative symptoms.

Sunde et al (2002)[45] investigated periapical microbiota with refractory apical periodontitis and found that approximately half (51%) of the bacterial strains were anaerobic. Gram positive species constituted 79% of the flora. Facultative organism such as *Staphylococcus, Enterococcus, Enterobacter, Pseudomonas, Stenotrophomonas, Sphingomonas, Bacillus* or *Candida* were recovered from 75% of the lesions and concluded that a wide variety of microorganisms, particularly Gram positive ones were found in the periapical lesions of the teeth with refractory apical periodontitis.

E.T.Pinheiro et al (2003)[23] conducted a study to identify the microbial flora within root canals of teeth with failed root canal treatment and to determine the association of various species with clinical features and concluded that the microbial flora in the root canals after the failure of root canal treatment were limited to a small number of predominantly gram positive microbial species. Facultative anaerobes, especially *E.fecalis* were the most commonly isolated microorganism. However, polymicrobial infection and obligate anaerobes were frequently found in canals of symptomatic root filled teeth.

E.T. Pinheiro et al (2003)[24] evaluated the microbiota of root filled teeth with persisting periapical lesion and to test the antibiotic susceptibility of the most prevalent species and found that the most frequently recovered genera are *Enterococcus, Streptococcus, Peptostreptococcus* and *Actinomyces* and concluded that microflora in canals after endodontic failure comprised predominantly facultative anaerobes and gram positive species and *E. faecalis* was the species most frequently isolated and showed erythromycin and azithromycin resistance among the isolates.

Isabelle Portenier et al(2003)[26] reviewed the different factors that make *E.faecalis* a potential problem in medicine and dentistry as it is a dominant microorganism in root filled teeth presenting with post treatment apical periodontitis and is rarely present in primary apical periodontitis.

Baumgartner JC et al (2004)[3] used PCR to detect the presence of specific species of bacteria in samples collected from two geographical locations and found out that there was significant difference in detection of bacteria between two geographical location for *Prevotella intermedia, P.nigrescens, P.tanerae, F.nucleatum and P.gingivalis* .

P.N.R Nair (2004)[19] reviewed the pathogenesis of apical periodontitis and causes of endodontic failure and noted that endodontic treatment has remarkably high degree of success. Nevertheless, endodontic treatment can fail. Most failures occur when treatment procedures, mostly of a technical nature, have not reached a satisfactory standard for the control and elimination of infection. Even when the highest standards and the most careful procedures are followed, failures still occur. This is because there are root canal regions that cannot be cleaned and obturated with existing equipments, materials and techniques and thus infection can persist. In very rare cases, there are also factors located within the inflamed periapical tissue that can interfere with post treatment healing of the lesion.

Isabela N. Rocas et al (2004)[28] undertook a study to determine possible associations between *E.faecalis* and different types of endodontic infection using nested PCR and concluded that *E.faecalis* is significantly more associated with asymptomatic ones. *E.faecalis* was much likely to be found in cases of failed endodontic therapy than in primary infections.

Gomes et al (2004)[11] investigated the root canal microbiota of primary and secondary root infected canals and the association of constituent species with specific endodontic signs and symptoms and found that individual canal root canal yielded a maximum of 10 bacterial species. Of the bacterial isolates, 70% were either strict anaerobes or microphilic. The anaerobes that were more frequently isolated were *Peptostreptococcus micros* (35%), *Fusobacterium necrophorum* (23.3%), *Fusobacterium nucleatum* (11.7%), *Prevotella intermedia* (16.7%), *Porphyromonas gingivalis* (6.7%), *Porphyromonas endodontalis* (5%). The root canal microflora of the untreated teeth with apical periodontitis was found to be mixed, comprising gram negative and gram positive and mostly anaerobic microorganisms and usually containing more than 3 species per canal. Whereas, facultative anaerobic and gram positive bacteria predominated the canals with failed endodontic treatment. It was also found that there was suggested relationship between anaerobes especially gram negatives and the presence or history of pain, tenderness to percussion and swelling.

JF Sequeira (2004)[36] investigated the occurrence of several microbial species in cases of failed endodontic therapy by means of

the polymerase chain reaction. He concluded microorganism in all cases of root filled teeth associated with periradicular lesions. *E.faecalis* was the most prevalent species, followed by other 4 anaerobic species *P.alactoltytics, P propionicum, D pneumosintes, F alocis.*

J.F. Sequeira (2004)[37] investigated the prevalence of 11 selected putative endodontic pathogens in the apical third of the infected root canals associated with periradicular lesions. The study results showed the presence of *Pseuramibacter alactolyticus* in 44 % of the cases examined, *Treponema denticola* in 26%, *F.nuleatum* in 26%, *P.endodontalis* in 17%, *Filifactor alocis* in 9%, *Dialister pneumosintes* in 4%, *P.gingivalis* in 4%, *T.forsythensis* in 4%. He concluded that occurrence of these bacterial species in the apical third of the infected root canals suggests that they can be involved in the causation of periradicular lesions.

Fouad et al (2005)[8] designed a study to identify *Enterococcus* spp in non- healing endodontic cases using PCR amplification and molecular sequencing and to determine if the prevalence of Enterococci is increased in diabetic patients and found that 8 out 37 specimens were positive for Enterococcus spp. Of these, 6(19%)

were from non-diabetic and 2 (33%) were from diabetic patients and concluded that *E.faecalis* was the only Enterococcal species detected with an overall prevalence of 22%.

J.F. Siqueira et al (2005)[38] investigated the prevalence of several uncultivated oral phylotypes, as well as newly named species in primary and persistent endodontic infections associated with chronic periradicular disease using nested PCR. The most prevalent species or phylotypes found in primary infections were *Dialister invisus, Synergistes oral clove, Olsenella uli*. Of the target bacteria only these three were found in persistent infections and concluded that detection of uncultivated phylotypes and newly named species in infected root canals suggest that these are previously unrecognised bacteria that may play a role in the pathogenesis of periradicular diseases.

J.F Sequeira et al (2005)[39] compared the prevalence of 7 putative endodontic pathogens in samples of primary endodontic infections from two distinct geographic locations and found that *Porphyromonas endodontalis* (79%), *Treponema denticola* (79%), and *Dialister pneumosintes* (76%) were the prevalent organisms in Brazilian samples. Whereas *Fusobacterium nucleatum* (38%),

Tannerella forsythia (26%) and *Treponema maltophilum* (24%) were predominantly seen in South Korean samples and concluded that prevalence of some species in infections of endodontic origin may significantly differ from one geographical location to another.

John M. Williams et al (2006)[46] compared real time quantitative PCR (qPCR) assay for *E.faecalis* detection and quantification during endodontic treatment, and a reverse – transcription PCR (RT- PCR) assay was also developed to detect the bacterium clinically in the viable but non-cultivable state (VBNC) and found that the bacterium is three times more prevalent in refractory than primary infections at each sample collection step. qPCR detected significantly more *E.faecalis* positive in samples than cultivation. VBNC *E.faecalis* was detected by RT PCR in four samples that were negatively cultivation that qPCR and RT PCR are more sensitive methods than cultivation for detection of *E.faecalis* in endodontic infections.

Brenda P.F.A Gomes et al (2006)[12] investigated the presence of *Enterococcus faecalis* in endodontic infections by culture and polymerase chain reaction analyses and found that culture and PCR detected the test species in 23 of 100 and 79 of 100 of the teeth,

respectively. *E faecalis* was cultured from 4% of the necrotic canal and from 42% of root treated canals. PCR detection identified the target species in 82% and 76% of primary and secondary infections respectively and concluded that *E.faecalis* was detected as frequently in teeth with necrotic pulps as in teeth with failing endodontic treatment when a PCR analysis was used.

G.O. Zoletic et al (2006)[47] evaluated the prevalence of *E.faecalis* in root filled teeth with or without periradicular lesions using PCR and cultivation methods and found that overall *E.fecalis* was detected by species specific 16 S rRNA gene based PCR in 40/50 teeth (80%) while culture revealed that occurence of this species in 8/50 teeth (16%). PCR was significantly more effective than culture in detecting *E.faecalis* species.

Pinheiro et al (2006)[25] designed a study to identify enterococcal species from canals of root filled teeth with periapical lesion using biochemical and molecular techniques and to investigate the genetic diversity of the isolates and found that *E.faecalis* was the only enterococcal species isolated from the canals of the root filled teeth with periapical lesions. Genetic heterogeneity was observed among the *E.faecalis* isolates following pulsed field gel

electrophoresis and sequence based typing methods and genetic diversity within the root canal strains was similar to previous reports regarding this species from different clinical and geographic origins.

Sedgley et al (2006)[34] compared the culture and real time quantitative PCR to detect and quantify in the same root canal sample and found that *E faecalis* was detected in 10.2% and 79.5% of the samples by culture and PCR respectively. *E faecalis* was detected more in retreatment cases than in primary samples and concluded that qPCR reported a significantly higher prevalence of *E faecalis* in endodontic samples than culture techniques.

L.C.N Brito et al (2007)[4] combined multiple displacement amplification (MDA) and checker board DNA - DNA hybridisation to examine the microbiota of endodontic infections and concluded that the endodontic that the endodontic microbiota was more complex than previously shown, although microbial profiles of the teeth with or without periradicular lesions did not differ significantly. Species commonly detected in endodontic samples included *Prevotella tannerae, Actinobacter baumanii* and *Prevotella oris.*

Brenda P.F.A Gomes et al (2007)[13] investigated the correlation between endodontic clinical signs and symptoms and the presence of *Porphyromonas gingivalis, Treponema denticola* and *Tannerella forsythia* or their association by nested PCR assay. *P.gingivalis, T.denticola, T.forsythia* were detected in 46%, 38% and 22 % of the symptomatic cases respectively. The bacterial complex composed by *P.gingivalis, T.denticola* and *T.forsythia* was found in 14% of the cases with spontaneous pain, tenderness to percussion, swelling and pain on palpation and concluded that high prevalence of these bacteria in the samples examined suggests that these bacteria are related to the aetiology of symptomatic periradicular diseases.

Ali Mahmoudpour et al (2007)[15] surveyed the incidence of *E.faecalis* infection in symptomatic and asymptomatic root canals of necrotic teeth using PCR. Using multiple cultivation dependent and PCR analysis, *E.faecalis* was found in 10% of samples and concluded that the results indicate that there is no significant difference in the incidence of *E.fecalis* between symptomatic and asymptomatic necrotic dental root canals.

Schizrrmeister et al (2007)[31] investigated the presence of microorganism by culture and polymerase chain reaction in

asymptomatic root filled teeth with periradicular lesions and found that prevalence of microorganism was 60% by culture and 65% by polymerase chain reaction.

Peciuliene V et al (2008)[22] reviewed on microorganisms in root canal infection and said that the composition of microflora of root canals differ in primary endodontic treatment and retreatment cases. Persistent disease in the periapical region after the root canal treatment presents a more complex situation as it was thought earlier.

Ribeiro et al (2011)[27] determined the bacterial diversity in primary endodontic infections by 16S rRNA sequence analysis and identified seventy phylotypes of which 6 were novel phylotypes belonging to the family Ruminococcaceae. The most prevalent taxa were *Atopium rimae* (50%), *Dialister invisus*, *Prevotella oris*, *Pseudoramibacter alactolyticus* and *Tannerella forsythia* (33%) and concluded that primary endodontic infection is characterized by a wide bacterial diversity which was predominantly represented by the phylum Firmicutes followed by Bacteroidetes.

Anderson AC et al (2012)[1] combined culture methods with culture-independent cloning methods to analyze the microbial flora of

root-filled teeth with periradicular lesions. Twenty-one samples from previously root-filled teeth were collected from patients with periradicular lesions. Microorganisms were cultivated, isolated and biochemically identified. Microorganisms were found in 12 samples with culture-dependent and -independent methods combined. The number of bacterial species ranged from 1 to 12 in one sample. The majority of the 26 taxa belonged to the phylum Firmicutes (14 taxa), followed by Actinobacteria, Proteobacteria and Bacteroidetes. One sample was positive for fungi, and archaea could not be detected. The results obtained with both methods differed. He concluded that combining the culture-dependent and independent approaches revealed new candidate endodontic pathogens and a high diversity of the microbial flora in root-filled teeth with periradicular lesions. Both methods yielded differing results, emphasizing the benefit of combined methods for the detection of the actual microbial diversity in apical periodontitis.

Materials and Methods

METHODOLOGY

MATERIALS

MATERIALS FOR COLLECTION OF CLINICAL SAMPLES (Fig:1)

30% Hydrogen peroxide (Leo pharma)

2.5% Sodium hypochlorite (Biolabs systems)

5% Sodium thiosulphate (Biolabs systems)

Saline solution (Nirlife heathcare)

Phosphate buffered saline.

Mueller Hinton Broth

MATERIALS / REAGENTS FOR PCR: (Fig:2)

Milli Q water

PCR buffer

dNTPs (Medox, India)

Taq DNA polymerase (Bangalore genei, India)

Ethedium bromide (Medox Biotech, India)

Agarose gel (Medox, India)

10x TAE buffer (Medox, India)

Ethidium bromide (Medox Biotech, India)

Gel loading dye

DNA ladder – 100 BP (Medox, India)

16S rDNA universal eubacterial primers (Sigma Aldrich)

Primers for identification of *Enterococcus faecalis* (Sigma Aldrich)

Primer Name	Primer Sequence and Genome position	Binding Spec.	Frag. Size	Targeting Site
Ef16F Ef16R	5'– AGAGTTTGATCCTGGCTCA-3' (POSITIONED AT 248466-83) 5'-GGTTACCTTGTTACGACTTC-3' (POSITIONED AT 249987-68)	Semi-specific	1522 bp	Full length coding sequence of 16S ribosomal RNA (4X per genome)
EfisF EfisR	5'-ATGCCGACATTGAAAGAAAAAATT-3' (POSITIONED AT 300261-84) 5'-TCAATCTTTGGTTCCATCTCT-3' (POSITIONED AT 301063-43)	Specific	803 bp	Coding region of iron sulphur binding protein
EfesF EfgsR	5'-GTGTTAAAACCATTAGGCGAT-3' (POSITIONED AT 112289 - 69) 5'-AAGCCTTCACGAACAATGG-3' (POSITIONED AT 11640-58)	Specific	650 bp	Coding region of GroES/EL chaperone protein

(Ali Mahmoudpour et al, 2007)

ARMAMENTARIUM:

CLINICAL AMAMENTARIUM (Fig.3)

- Diagnostic Instruments: Mouth Mirror, Explorer, Tweezer.
- Lignox A (2 % lignocaine with 1:80,000 adrenaline)(Indoco Remedies)
- Disposable Syringes (Unolock , HMD Ltd)
- Rubber dam (Dental Dams, Sg, Malaysia)
- Spoon Excavator
- Airotor Hand Piece (PanaAir , NSK)
- Access cavity burs (no.2,no.4 round bur, safe tip tapered diamond) (Mani.Inc)
- Apex locator (Root ZX Mini , J morita , Japan)
- Gates Glidden drills (Mani, inc)
- K-type files (Mani , inc)
- Hedstrom files (Dentsply Maillefer)
- Absorbent paper points (Dentsply Maillefer)
- Eppendorf tubes

LAB ARMAMENTARIUM

DNA ISOLATION AND PURIFICATION:

- Eppendorf tubes (Eppendorf, Germany)
- -20^0 C freezer (Rands instruments, India)
- Micropipette (Eppendorf,Germany)
- Micropipette tips (Tarsons)

POLYMERASE CHAIN REACTION (Fig. 8,9,10)

- PCR tubes
- Micropipette (Eppendorf, Germany)
- Microcentrifuge (Spinwin)
- Eppenndorf tubes (Eppendorf , Germany)
- PCR thermal cycler (Eppendorf Master Cycler Gradient , Germany)

AGAROSE GEL ELECTROPHORESIS (Fig.11)

- Gel tray
- Gel comb
- Cello tape
- Electrophoresis tank with power supply
- UV transilluminator

- Microwave oven (Godrej)
- Geldoc (Biorad Gel Documentation System)

SOURCE OF THE DATA

The study was approved by the Ethical Committee of Ragas Dental College and Hospital and due clearance was obtained for carrying out the investigation. A total number of 40 cases were selected from those patients who were referred to the Department of Conservative Dentistry and Endodontics, Ragas Dental College and Hospital for root canal therapy. An informed consent was signed by all the patients participating in the study.

All the selected patients were subjected to clinical and radiographical examination.

METHOD OF COLLECTION OF DATA (INCLUDING SAMPLING PROCEDURE)

INCLUSION CRITERIA

Subjects willing to participate in the study were selected with the following inclusion criteria.

- Both males and females aged between 20-65 years were included.
- Only immunocompetent subjects were included.
- Teeth with patent canals.(verified using pre operative radiographs)

EXCLUSION CRITERIA:

1. Systemic diseases
2. Use of any antibiotics in past 3 months
3. Pregnancy and lactation
4. Immunocompromised patients
5. Participation in other clinical study during previous 3 months
6. Teeth that cannot be isolated with rubber dam
7. Teeth exhibiting frank exposure of the root filling material to the oral cavity in group 2 cases.
8. Calcified canals (checked using radiographs in 2 angles)
9. Tortuous canals (checked using radiographs in 2 angles)
10. Canals with separated instruments (checked using radiographs)

11. Root fracture (checked using radiographs using horizontal and vertical angulations)
12. Teeth with developmental defects
13. Teeth having periodontal pockets greater than 4mm deep.

According to the above inclusion and exclusion criteria, the 40 subjects who were selected for the study were divided into two groups, with each group consisting of 20 subjects.

Group 1:

- Patients with diagnosis of primary endodontic infection in any teeth.

Group 2:

1. Patients requiring retreatment of endodontically treated teeth with a diagnosis of apical periodontitis.
2. Patients who had undergone endodontic therapy more than 2 years ago.
3. All the root filled teeth that were symptomatic and had radiographic evidence of periradicular disease.
4. Root filled teeth with coronal seal.

5. The terminus of the root canal fillings was at least 2mm short of the radiographic apex.

SAMPLING PROCEDURE:

Each tooth that was sampled was cleansed with pumice and isolated with a rubber dam. Samples were obtained under strict asepsis. The tooth and the surrounding field was disinfected using 30% hydrogen peroxide followed by 2.5% sodium hypochlorite for 30 seconds. The sterility of the operating field was checked after inactivation of the antiseptic solution using 5% sodium thiosulphate in order to avoid interferences with the results. Endodontic access was established using sterile burs (no.2, no.4 round burs) in group 1 cases. A sterile 15 size K file was introduced in to the root canal holding the file with the sterile lock pliers. Working length was determined 1mm short of the apex using apex locator and the same was confirmed with radiographs. Following this, a sterile H file was introduced in to the root canal and the inner walls of the root canal was filed and with its handle cut off was immediately transferred to the Eppendorf tube containing phosphate buffered saline.

In group 2 cases, the same disinfection protocol was followed as previously described. The existing coronal restoration was removed using sterile burs (no.4 round bur), the pre existing root canal filling was removed using sterile Gates Glidden drills(size 2,3) and H files (size 25, 30) without the use of any chemical solvents. Working length was determined in the same way as in group 1 cases. Following this , a sterile H file was introduced in to the root canal, the inner walls of the root canal was filed and after the handle of the file was cut off, it was immediately transferred to the Eppendorf tube containing phosphate buffered saline.

Sampling included single root canal, even in the case of multi rooted teeth in order to confine the microbiological evaluation to a single ecological environment. The criteria used to choose the canal to be microbiologically investigated in the multi rooted teeth were the presence of exudation, or in its absence, the largest canal, or the canal associated with periapical radiolucency. Before sampling the selected canals of the multi rooted teeth, the entrance of the others were closed with sterile cotton pellets.

Samples once collected, were submitted to the Department of Microbiology, Balaji Science and Research Institute within 2 hours for PCR analysis

DNA EXTRACTION

The collected clinical samples were brought to room temperature and centrifuged. The supernatant was discarded. To the deposit sterile Milli - Q water was added, vortexed, boiled for 10 minutes and micro centrifuged at 10,000 rpm for 3 minutes. Then the supernatant was stored at -20 ºC till assay. Ten microlitre of the supernatant was directly used as template for PCR assay.

DIRECT SCREENING OF CLINICAL SAMPLES BY NESTED PCR

Nested PCR was performed using *16S rDNA* universal eubacterial primers to screen for the bacterial species in the root canal samples. The PCR reaction mixture of 50 µl volume consisted of 1 unit of *Taq* DNA polymerase (Bangalore genei, India.), 5 µl of 10X PCR buffer, 0.5 µM of each primer (Sigma-Aldrich Pvt Ltd, India), 0.2 mM of each dNTP (Medox Biotech India Pvt Ltd, India) and 5µl of DNA template. 1µl of the first round amplified product was used

as DNA template in the second round of amplification. Ten microlitres of each reaction product was mixed with 10 µl of 2× loading buffer and fractionated in a 1.5 % agarose gel electrophoresis with Tris-Borate EDTA buffer containing ethidium bromide (0.5 µg /ml(Medox Biotech India Pvt Ltd, India), using a 100 bp DNA ladder (Medox Biotech India Pvt Ltd, India) as a size marker.

DETECTION OF *Enterococcus faecalis* by MULTIPLEX PCR

The PCR reaction mixture of 25 µl volume consisted of 1 unit of *Taq* DNA polymerase (Bangalore genei, India.), 5 µl of 10X PCR buffer, three pairs of primers each of 0.5 µM of each primer (three) (Sigma-Aldrich Pvt Ltd, India), 0.2 mM of each dNTP (Medox Biotech India Pvt Ltd, India) and 5µl of DNA template.

PCR THERMOCYCLING PROGRAMME

THERMAL CYCLING CONDITIONS FOR MULTIPLEX PCR

1. Initial denaturation step at 95 ◦C for 4 minutes followed by 35 cycles of

Denaturation at 95^{o}C for 30 seconds

Primer Annealing at 58^{o}C for 30 seconds

Extension at 72^{o}C for 1.30 min and

2. Final extension step at 72◦C for 10 minutes.

THERMAL CYCLING CONDITIONS FOR NESTED PCR

First Round

1. Initial denaturation step at 94 ^{o}C for 1.30 minutes followed by
2. 41 cycles of

   Denaturation at 94^{o}C for 30 seconds

   Primer Annealing at 50^{o}C for 30 seconds

   Extension at 72^{o}C for 1 min and
3. Final extension step at 72◦C for 10 minutes

Second Round

1. Initial denaturation step at 94 ^{o}C for 1.30 minutes followed by
2. 31 cycles of

   Denaturation at 94^{o}C for 30 seconds

   Primer Annealing at 50^{o}C for 30 seconds

   Extension at 72^{o}C for 1 min and
3. Final extension step at 72◦C for 10 minutes.

GEL ELECTROPHORESIS FOR DETECTION OF PCR AMPLICON

The PCR products were fractionated in a 1.5% Agarose gel electrophoresis.

REAGENTS REQUIRED

1. Preparation of TBE Buffer (1x)

490 ml of double distilled water

10 ml of 50 x TBE Buffer

2. Ethidium bromide

Ethidium bromide - 10 mg

Distilled water - 1 ml

PROCEDURE

PREPARATION OF 1.5% AGAROSE GEL

1.5 grams of agarose was weighed and transferred into 250 ml conical flask containing 100 ml of 1x TBE buffer. The agarose was dissolved by boiling in a microwave oven.

The appropriate sized gel tray and comb was washed. Cello tape was fixed on both sides of the tray. The comb was placed on the gel tray without touching the bottom and left on an even surface.

Agarose was cooled down, 0.5 µl of ethidium bromide was added and mixed well. It was poured on the gel tray and allowed to polymerize.

PREPARATION OF SAMPLE AND LOADING

TBE buffer (0.5 x) was added to the electrophoresis tank to a level for the gel to be immersed. The cello tape was removed from the gel tray and the tray was placed in the electrophoresis tank. The comb was carefully removed from the gel tray.

Ten microlitre of the PCR product was mixed with 10 µl of 2x gel loading buffer and loaded into the wells. The electrodes were connected. The power was switched ON and set at 100 V. After the completion of the electrophoresis, gel was taken to the transilluminator and observed under UV-light for documentation. (Biorad gel documentation)

INTERPRETATION:

100 bp DNA ladder (MEDOX) was used as a size marker and sterile milli Q water was used as blank control.

NUCLEOTIDE SEQUENCE ANALYSIS

The amplicon size of first round PCR was 766bp and the second round PCR was 470bp. The second round product was further sequenced. All the *16S-rDNA* sequences obtained were blasted in the

Genbank database. In addition, all *16S-rDNA* sequences were compared with the database sequences of the Ribosomal Database Project and the Human Oral Microbiome Database.

PROCEDURAL SEQUENCE FOR IDENTIFICATION OF MICROORGANISM BY PCR

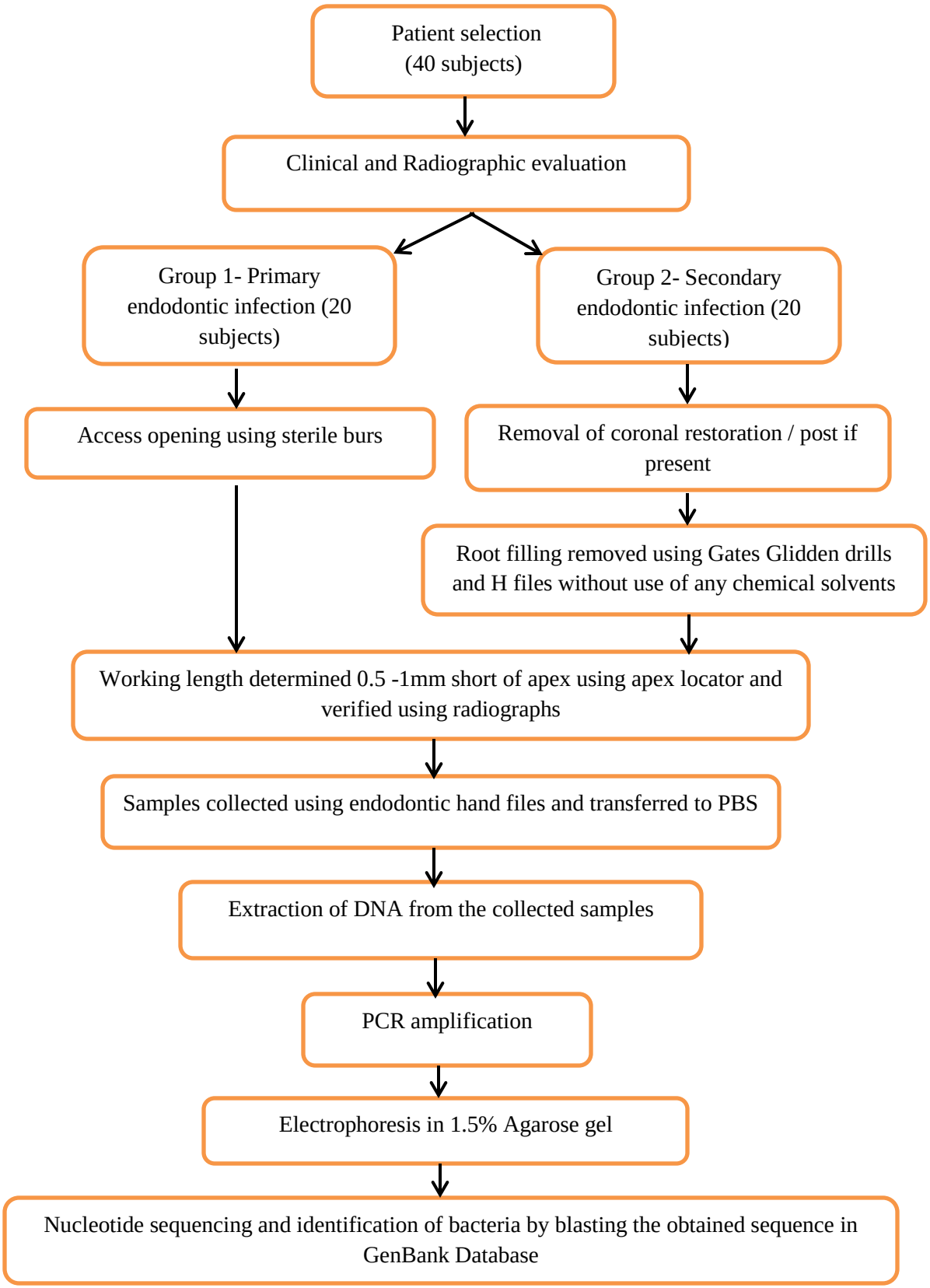


Fig 1: Materials for collection of clinical samples

Fig 2: Materials, Reagents, Primers for PCR analysis

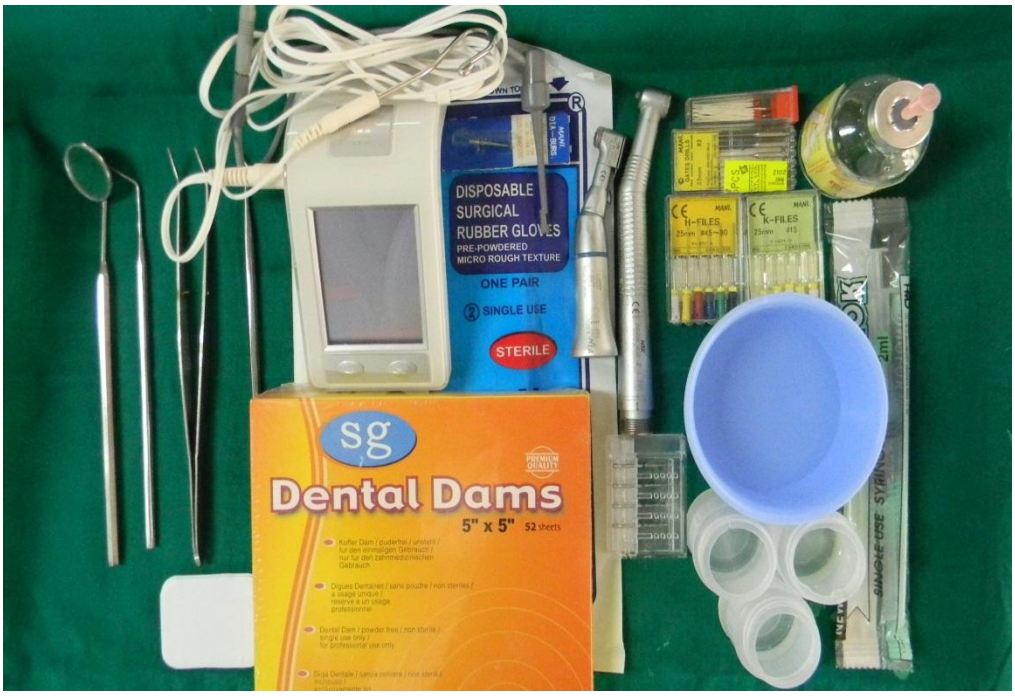


Fig 3: Clinical armamentarium

Fig 4: Isolation of the tooth to be sampled and disinfection of the surrounding field.

Fig 5: Sample collected from root canal using hand file

Fig 6: Collected samples transferred to PBS

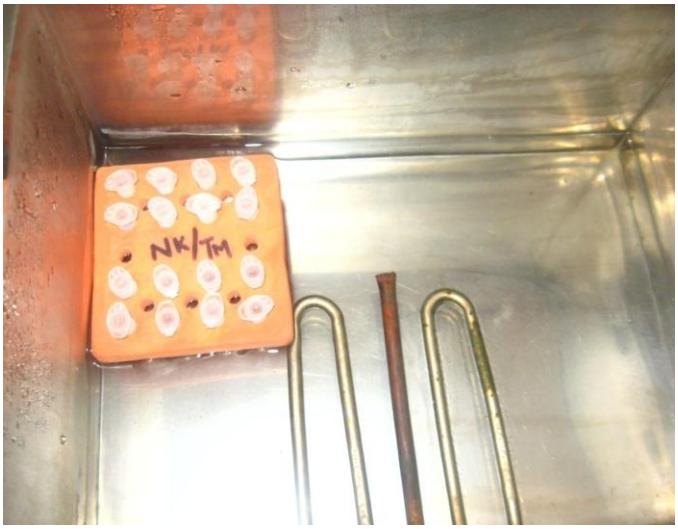


Fig 7: DNA extraction by boiling lyses method

Fig 8: Mini Centrifuge

Fig 9: PCR mixture

Fig 10: PCR Thermal Cycler

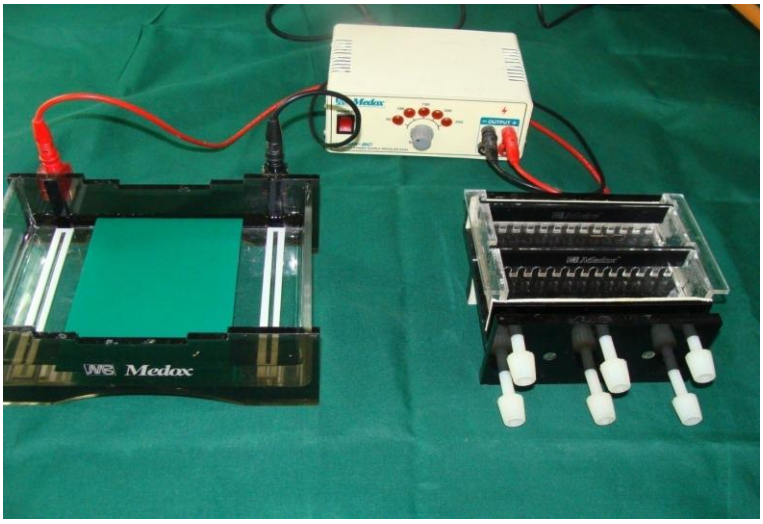


Fig 11: Gel Electrophoresis Unit

Fig 12: Gel Documentation Unit

Results

Table 1: Microorganisms isolated from Primary Endodontic Infection (Group 1)

Case no.	Sex	Age	Tooth no.	TTP	PRL	WPL	UP	Microorganisms Isolated
B1	F	34	11	Y	Y	Y	$+^{ve}$	*Lysinibacillus fusiformis, Actinomyces naeslundi*
B2	F	41	11	Y	Y	Y	$+^{ve}$	*Bacteroidetes bacterium, Acinetobacter baumannii*
B3	F	25	21	Y	N	Y	$+^{ve}$	*Acinetobacter baumnnii, Bacteroidetes oral clone, Enterobacter cancerogenus*
B4	M	27	41	Y	Y	Y	$+^{ve}$	*Enterobacter cloacae, Microbacterium* spp
B5	M	28	42	Y	Y	Y	$+^{ve}$	*Prevotella heparinolytica, Lysinibacillus fusiformis*
B6	F	39	21	Y	Y	Y	$+^{ve}$	*Lactobacillus* spp, *Peptostreptococcus* spp
B7	M	39	21	Y	Y	Y	$+^{ve}$	*Fusobacterium* spp, *Bacteroidetes* spp, *Porphyromonas* spp
B8	M	26	13	Y	N	Y	$+^{ve}$	*Prevotella* spp, *Actinomyces odontolyticus*
B9	M	48	23	Y	Y	Y	$+^{ve}$	*Enterococcus faecalis, Actinomyces* spp
B10	F	41	36	Y	Y	Y	$+^{ve}$	*Prevotella heparinolytica, Peptostreptococcus* spp
B11	M	40	24	Y	Y	Y	$+^{ve}$	*Actinomyces naeslundi, Enterobacter* spp, *Lactobacillus* spp
B12	M	22	46	Y	Y	Y	$+^{ve}$	*Enterobacter* spp, *Prevotella* spp, *Acinetobacter baumannii*
B13	F	29	14	Y	N	Y	$+^{ve}$	*Bacteroidetes* spp, *Porphyromonas* spp
B14	M	38	21	Y	Y	Y	$+^{ve}$	*Lactobacillus acidophilus, Streptococcus sanguis*
B15	F	33	24	Y	Y	Y	$+^{ve}$	*Bacteroidetes bacterium, Actinomyces* spp
B16	F	31	22	Y	Y	Y	$+^{ve}$	*Microbacterium spp, Enterobacter cloacae*
B17	M	46	21	Y	Y	Y	$+^{ve}$	*Peptostreptococcus* spp, *Porphyromonas gingivalis*
B18	M	27	22	Y	N	Y	$+^{ve}$	*Campylobacter* spp, *Treponema denticola, Prevotella* spp
B19	M	33	13	Y	Y	Y	$+^{ve}$	Porphyromonas spp, Bacteroidetes oral clone
B20	M	31	11	Y	Y	Y	$+^{ve}$	Enterobacter spp, Actinomyces naeslundi, Fusobacterium spp.

TTP- Tender to Percussion, Y-Yes , N-No ; PRL- Presence of Periapical Radiolucency,Y-Yes, N-No; WPL-Widening of Periodontal Ligament, Y-Yes, N-No ; UP- Universal Primer.

Table 2: Microorganisms isolated from secondary endodontic infection (Group 2)

Case no.	Sex	Age	Tooth no.	TTP	PRL	RF (in mm)	UP	Microorganisms Isolated
A1	M	33	11	Y	Y	2	$+^{ve}$	*Escerichia coli, Actinomyces* spp, *Prevotella* spp
A2	M	29	22	Y	Y	3	$+^{ve}$	*Bacillus subtilis, Fusobacterium* spp
A3	M	45	11	Y	Y	2	$+^{ve}$	*Prevotella heparinolytica, Streptococcus* spp
A4	F	42	22	Y	Y	3	$+^{ve}$	*Enterobacter hormaechei, Fusobacterium nucleatum*
A5	F	29	12	Y	Y	2	$+^{ve}$	*Enterococcus faecalis, Actinomyces* spp
A6	M	31	23	Y	Y	3	$+^{ve}$	*Enterococcus faecalis, Bacillus subtilis*
A7	M	43	46	Y	Y	2	$+^{ve}$	*Actinomyces spp, Streptococcus mitis*
A8	F	28	37	Y	Y	2	$+^{ve}$	*Butyrivibrio* spp
A9	M	37	41	Y	Y	3	$+^{ve}$	*Lactobacillus paracasei, Clostridium* spp, *Porphyromons* spp
A10	M	38	31	Y	Y	3	$+^{ve}$	*Fusobacterium nucleatum, Lactobacillus* spp
A11	F	34	24	Y	Y	2	$+^{ve}$	*Propionibacterium* spp, *Streptococcus* spp
A12	F	27	33	Y	Y	4	$+^{ve}$	*Enterococcus faecalis*
A13	F	47	32	Y	Y	2	$+^{ve}$	*Actinomyces* spp, *Prevotella* spp
A14	M	27	21	Y	Y	3	$+^{ve}$	*Enterobacter hormachei, Eubacterium* spp
A15	F	35	36	Y	Y	2	$+^{ve}$	*Streptocossus mitis, Bifidobacterium* spp
A16	M	30	36	Y	Y	2	$+^{ve}$	*Enterococcus faecalis, Prevotella* spp
A17	M	46	11	Y	Y	4	$+^{ve}$	*Actinomyces naeslundi, Streptococcus sanguis*
A18	F	41	13	Y	Y	3	$+^{ve}$	*Fusobacterium* spp, *Bifidobacterium* spp
A19	M	32	12	Y	Y	2	$+^{ve}$	*Veilonella* spp, *Streptococcus anginosus*
A20	F	44	31	Y	Y	2	$+^{ve}$	*Porphyromonas* spp, *Campylobacter* spp, *Propionobacterium* spp.

TTP- Tender to Percussion, Y-Yes; PRL- Presence of Periapical Radiolucency,Y-Yes; RF– Apical limit of Root filling; UP- Universal Primer

Table 3: Classification of microorganisms identified in 20 cases diagnosed with Primary endodontic infection

MICROORGANISM	Gram Staining	Requirement of O_2	Phylum	% present out of 20 cases
Bacteroidetes spp	$-^{ve}$	Anaerobe	Bacteroidetes	30%
Actinomyces	$+^{ve}$	Anaerobe (f)	Actinobacter	30%
Enterobacter spp	$-^{ve}$	Anaerobe (f)	Proteobacteria	30%
Prevotella spp	$-^{ve}$	Anaerobe	Bacteroidetes	25%
Porphyromonas	$-^{ve}$	Anaerobe	Bacteroidetes	20%
Acinetobacter spp	$-^{ve}$	Aerobe	Proteobacteria	15%
Lactobacillus spp	$+^{ve}$	Anaerobe (f)	Firmicutes	15%
Peptostreptococci	$+^{ve}$	Anaerobe	Firmicutes	15%
Fusobacterium	$-^{ve}$	Anaerobe	Fusobacteria	10%
Lysinibacillus fusiformis	$+^{ve}$	Anaerobe (f)	Firmicutes	10%
Microbacterium spp	$+^{ve}$	Aerobe	Actinobacteria	10%
Campylobacter spp	$-^{ve}$	Anaerobe	Proteobacteria	5%
Streptococcus spp	$+^{ve}$	Aerobe (f)	Firmicutes	5%
Enterococcus faecalis	$+^{ve}$	Anaerobe (f)	Firmicutes	5%
Treponema denticola	$-^{ve}$	Anaerobe	Spirochaetes	5%

(f) - facultative

Table 4: Classification of microorganisms identified in 20 cases diagnosed with secondary endodontic infection

MICROORGANISM	Gram Staining	Requirement of O_2	Phylum	% present out of 20 cases
Streptococcus spp	$+^{ve}$	Aerobic (f)	Firmicutes	30%
Actinomyces	$+^{ve}$	Anaerobe (f)	Actinobacteria	25%
Enterococcus faecalis	$+^{ve}$	Anaerobe (f)	Firmicutes	20%
Fusobacterium spp	$-^{ve}$	Anaerobe	Fusobacteria	20%
Prevotella spp	$-^{ve}$	Anaerobe	Bacteroidetes	20%
Porphyromonas spp	$-^{ve}$	Anaerobe	Bacteroidetes	10%
Lactobacillus spp	$+^{ve}$	Anaerobe (f)	Firmicutes	10%
Enterobacter spp	$-^{ve}$	Anaerobe	Proteobacteria	10%
Bacillus subtilis	$+^{ve}$	Aerobe	Fimicutes	10%
Propionibacterium spp	$+^{ve}$	Anaerobe (f)	Actinibacteria	10%
Bifidobacterium spp	$+^{ve}$	Anaerobe	Actinobacteria	10%
Eubacterium spp	$-^{ve}$/ $+^{ve}$	Anaerobe	Firmicutes	5%
Escherichia coli	$-^{ve}$	Anaerobe (f)	Proteobacteria	5%
Campylobacter spp	$-^{ve}$	Anaerobe (f)	Proteobacteria	5%
Clostridium spp	$+^{ve}$	Anaerobe (f)	Fermicutes	5%
Butyrivibrio spp	$+^{ve}$	Anaerobe	Firmicutes	5%
Veilonella spp	$-^{ve}$	Anaerobe (f)	Fermicutes	5%

(f)- facultative.

RESULTS

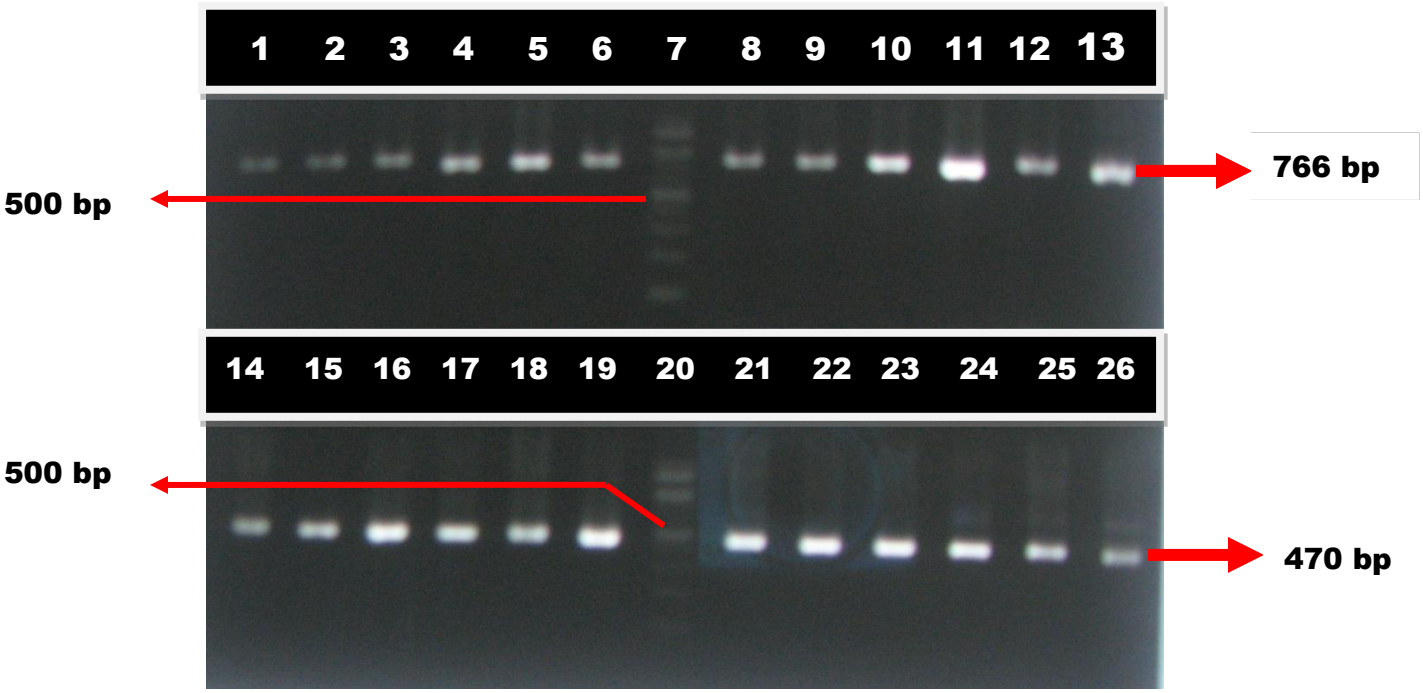
Table 1 shows the occurrence of 15 various bacterial genera in 20 cases investigated which were grouped as primary endodontic infections. The bacterial genera that were found are as follows. *Bacteroidetes* spp, *Actinomyces, Enterobacter* spp, *Prevotella* spp, *Porphyromonas* spp, *Acinetobacter* spp, *Lactobacillus* spp, *Peptosreptococcus* spp, *Fusobacterium* spp, *Lysinibacillus fusiformis, Microbacterium* spp, *Campylobacter* spp, *Streptococcus* spp, *Enterococcus faecalis* and *Treponema denticola*.

Table 2 shows the occurrence of 17 various bacterial genera in 20 cases investigated which were grouped as secondary endodontic infections. The bacterial genera that were found are as follows. *Streptococcus* spp, *Actinomyces* spp, *Enterococcus faecalis, Fusobacterium* spp, *Prevotella* spp, *Porphyromonas* spp, *Lactobacillus* spp, *Enterobacter* spp, *Bacillus subtilis, Propionibacterium* spp, *Bifidobacterium* spp, *Eubacterium* spp, *Escherchia coli, Campylobacter* spp, *Clostridium* spp, *Butyrivibrio* spp *and Veilonella* spp.

Table 3 shows the classification of the identified bacteria in primary endodontic infection according to their phyla, gram staining and oxygen requirement. The bacteria identified were classified in to 6 phyla namely the Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria and Spirochaetes. The majority of the bacteria found were anaerobic.

Table 4 shows the classification of the identified bacteria in secondary endodontic infection according to their phyla, gram staining and oxygen requirement. The bacteria identified were classified in to 5phyla namely Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria and Fusobacteria. Majority of the bacteria identified were facultative anaerobes

Image 1: GEL PHOTOGRAPH (PRODUCTS OF NESTED PCR)

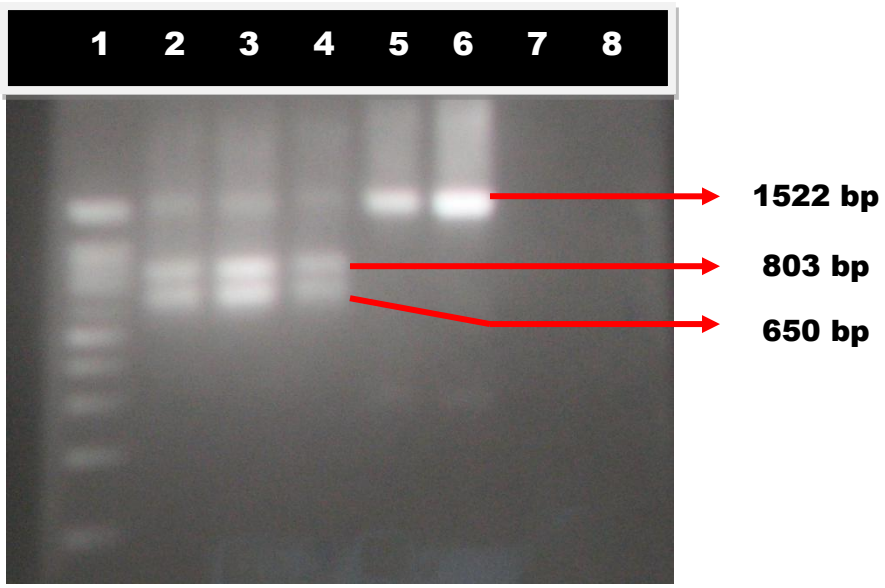


Lane 1-6, 8-13 – amplicons of first round

Lane 14- 19, 21-26 - amplicons of second round

Lane 7, 20 – 100 bp Ladder

Image 2: GEL PHOTOGRAPH (PRODUCTS OF MULTIPLEX PCR FOR IDENTIFICATION OF *E.FAECALIS*)



Lane 1- 100 bp ladder

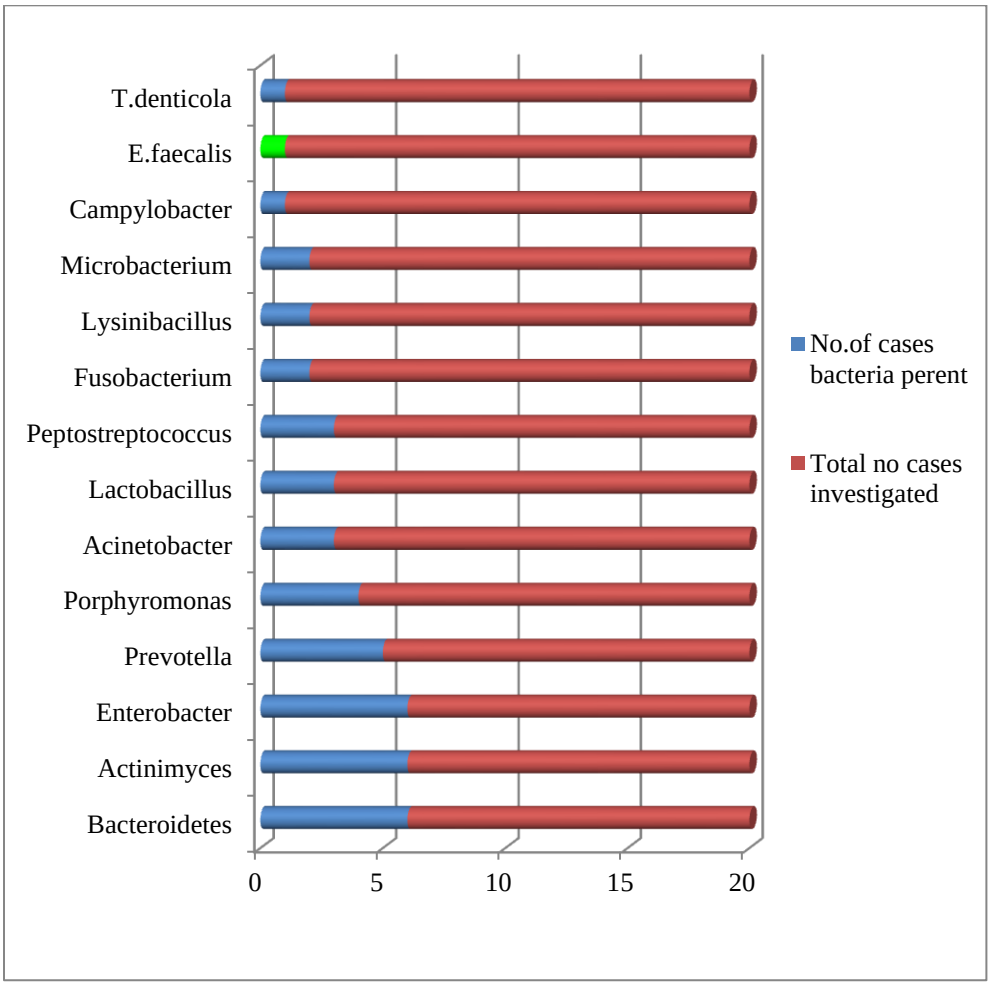
Lane 2 – Positive control (*E.faecalis* ATCC 29212)

Lanes 3- 5 - Clinical sample

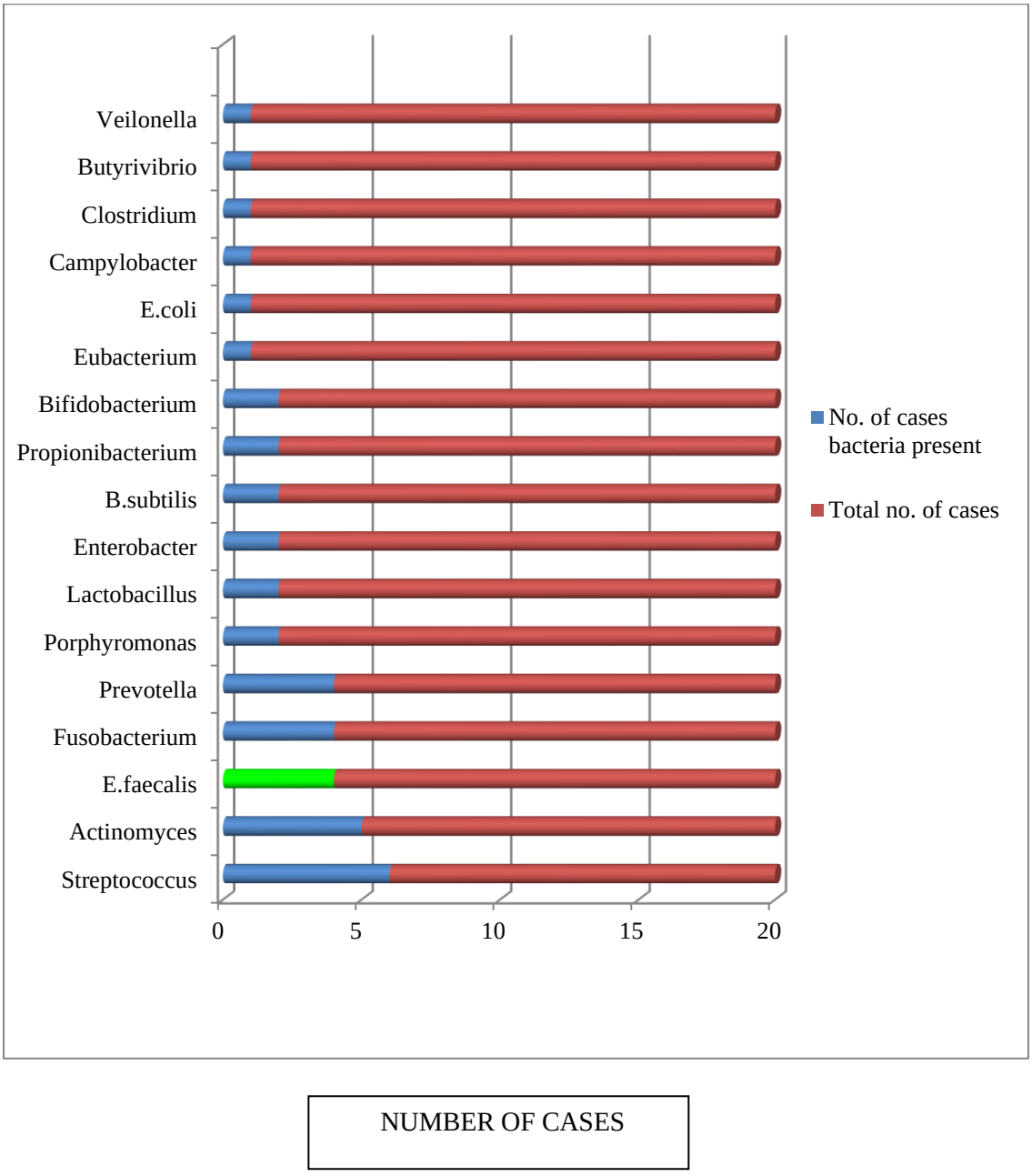
Lane 6- Negative control

Lane 7- blank control

GRAPH 1: Microorganisms present in total number of cases examined under Group 1(total no. cases examined:20)



GRAPH 2: Microorganisms present in total number of cases examined under Group 2 (total no. of cases examined: 20)



Discussion

DISCUSSION

The rationale of endodontic treatment is to eradicate the microorganisms and to prevent it from repopulating the pulpal and periapical spaces. Eliminating the microorganisms that invade and colonize the root canal space still remains to be one of the greatest challenges even today despite of tremendous advancements in the field of endodontics. The endodontic literature is replete with evidences from the earnest work of eminent investigators proving the fact that, endodontic infections are characterized by consortium of microorganisms and their presence has a direct influence on the treatment outcome.

It is a known fact that, more than 700 bacterial species are recognized as components of the oral microflora[20]. However, relatively only few species had shown evidences of invading the root canal and producing infections. Although all varieties of microorganisms found in the oral microbiota has equal chance of invading the pulp tissue and root canal, only those that could withstand this drastic environment do survive.

Microorganisms that are encountered in the infected root canals are most often found in various combinations rather than a single species. They present themselves in two forms, either as Planktonic form consisting of free floating bacteria or as Biofims which are dense aggregates of microbes adhering to canal walls leading to bacterial condensation. This concept of biofilms was a breakthrough in endodontic microbiology and has paved way in better understanding of endodontic infections, especially those of persistent variety[1].

Root canal infection can be broadly classified as primary endodontic infection and secondary endodontic infection. Primary endodontic infection deals with the untreated infected root canals where microorganisms access and colonize the pulp tissue causing its functional impairment. Secondary endodontic infection deals with the failure of endodontic treatment, especially due to persistence of microbial infection in the root canal system.

Over the years, considerable research has focused on the composition of microbiota of the root canal system. Results of such studies have clearly defined the microbial differences between the

primary and secondary endodontic infections.[35-41] This is due to the fact that, there is change in the root canal environment namely, the type and availability of nutrients, oxygen tension and bacterial interactions.[12,23,24]

Since, the characterization of the microbial communities infecting the endodontic system in each clinical condition might help in establishing a correct prognosis and a definite treatment strategy, identification of bacteria in both primary and secondary endodontic infection was undertaken in this study.

Although there are myriad studies done in the western countries which tabulate the microorganisms that are predominantly present in endodontic infections, the microorganisms which are prevalent in the root canals of the patients in some geographic region need not be the same in other geographic location which was the proposed hypothesis of the present study.

Since endodontic infections are regarded as endogenous infections caused by the members of the oral microbiota, it is conceivable that any differences in the latter will ultimately have its influence on the composition of the endodontic microbiota.[39] It has been postulated that

some host and environmental related factors might have its influence in certain microbial species colonizing the oral cavity. This might probably be responsible for the differences in the composition of the microbial community.[39]

Genetic predisposition and differential exposure to environmental conditions such as climatic conditions, quality of community water supplies, feeding habits, rate of individual infected by the same species within the communities, physiological stress, access to and frequency of dental care and educational factors all can influence the variation in oral ecology.[39]

As there was always paucity of information regarding isolation and identification of endodontic pathogens in India, this study was designed to isolate and identify microorganisms present in both primary and secondary endodontic infections pertaining to Indian population.

When the study was aimed to identify the endodontic microflora, it deemed the necessity to adopt latest method of microbial identification through molecular genetic methods which was proven to

be more sensitive and specific. Thus, PCR which had a cutting edge over the traditionally followed culture methods was opted in this study.

The molecular genetic methods were not only able to detect cultivable species but also uncultivable microbial species and strains. They were sensitive, highly specific and accurately identified the microbial strains with ambiguous phenotypic behavior. They were faster and less time consuming and most importantly they do not require carefully controlled anaerobic conditions during sampling and transportation which was advantageous since fastidious anaerobic bacteria and other fragile microorganisms might lose viability during transit.[40]

Microbiological analysis of root canal flora in primary endodontic infection was always felt easier due to large amount of bacterial cells and species in the root canal. But, it is entirely a different scenario as far as secondary infection was considered because in cases being retreated. The accessible organism in the root canal can be low and a number of microbial cells can also be lost during the procedures that were attempted to remove the root canal filling. As a result, the number of

cells sampled can fall short of the detection rate and the prevalence of a given species might be underestimated.[39] So, this again demands a technique that could be highly sensitive and specific. Therefore in this present study, a metagenomic approach by Nested and Multiplex PCR was carried out to identify the bacteria present in both primary and secondary endodontic infection.

The study was approved by the Ethical committee of Ragas dental college and hospital and due clearance was obtained for carrying out the investigation. A total of 40 cases were selected from those patients who were referred to the Department of Conservative Dentistry and Endodontics, Ragas dental college and Hospital for root canal therapy. An informed consent was signed by all the patients participating in this study.

Patients in the age group between 20 and 65 years who were immunocompetant and do not have any systemic diseases were selected for the study. Teeth with patent canals that were verified using preoperative radiographs only were selected. Teeth having calcified canals, tortuous canals, root fractures all of which were checked using

radiographs were excluded from the study. Teeth that could not be isolated with rubber dam were excluded from the study. In retreatment cases, teeth exhibiting frank exposure of the root filling material to the oral cavity, separated instruments were excluded from the study. Teeth having periodontal pockets greater than 4 mm were excluded since there can be possible interferences by periodontal pathogens. Patient who had taken antibiotics within the last 3 months were excluded from the study.

History, clinical examination and diagnostic procedures like thermal and electric pulp sensibility tests, intra oral periapical radiographs were used to conclude the status of the pulp and periapical tissues. In accordance with the above, the selected 40 patients were grouped in to two groups consisting of 20 subjects in each.

Group 1 – Any tooth with the diagnosis of primary endodontic treatment

Group 2 – Failed root canal treated tooth requiring retreatment.

In collecting the microbial samples, utmost care was taken to avoid any means of cross contamination. Each tooth was sampled cleansed with pumice and isolated with rubber dam. The tooth and the

surrounding field was disinfected using 30% hydrogen peroxide followed by 2.5% sodium hypochlorite for 30 seconds. The sterility of the operating field was checked after inactivation of the antiseptic solution using 5% sodium thiosulphate in order to avoid interferences with the results. Endodontic access was established using sterile burs (no.2, no.4 round burs) in group 1 cases. A sterile 15 size K file was introduced in to the root canal holding the file with the sterile lock pliers. Working length was determined 1mm short of the apex using apex locator and the same was confirmed with radiographs. Following this, a sterile H file was introduced in to the root canal and the inner walls of the root canal was filed and with its handle cut off was immediately transferred to the Eppendorf tube containing phosphate buffered saline.

In group 2 cases, the same disinfection protocol was followed as previously described. The existing coronal restoration was removed using sterile burs (no.4 round bur) under high vacuum suction, the preexisting root canal filling was removed using sterile Gates Glidden drills(size 2,3) and H files (size 25 ,30) without the use of any chemical solvents. Working length was determined in the same way as in group 1

cases. Following this , a sterile H file was introduced in to the root canal, the inner walls of the root canal was filed and after the handle of the file was cut off, it was immediately transferred to the Eppendorf tube containing phosphate buffered saline.

Sampling included single root canal, even in the case of multi rooted teeth in order to confine the microbiological evaluation to a single ecological environment. The criteria used to choose the canal to be microbiologically investigated in the multi rooted teeth were the presence of exudation, or in its absence, the largest canal, or the canal associated with periapical radiolucency. Before sampling the selected canals of the multi rooted teeth, the entrance of the others were closed with sterile cotton pellets. Samples once collected, were submitted to the Department of Microbiology, Balaji Science and Research Institute within 2 hours for PCR analysis.

Boiling and lyses method was followed for extraction of DNA from the collected clinical samples. The samples were brought to room temperature and centrifuged. The supernatant was discarded. To the deposit sterile Milli - Q water was added, vortexed, boiled for 10

minutes and micro centrifuged at 10,000 rpm for 3 minutes. Then the supernatant was stored at -20 ºC till assay. Ten microlitre of the supernatant was directly used as template for PCR assay.

PCR was carried out in discrete cycles and each cycle of amplification can, if 100 % capable doubles the amount of target DNA. The target DNA is exponentially amplified such that after n cycles, there is 2n times as much target DNA as was present initially. The basic procedure of PCR includes repeated cycles of amplifying selected nucleic acid sequences.

Each cycle consists of three steps

1. Denaturation, in this step, double strands of the target DNA are separated
2. A primer annealing step, performed at a lower temperature, in which primers anneal to their complementary target sequences
3. In the extension reaction step, DNA polymerase extends the sequences between the primers.

This was further subjected to Nested PCR for direct screening. Nested polymerase chain reaction was performed with two sets of

primers, used in two successive runs of polymerase chain reaction. The larger fragment produced by the first round of PCR is used as the template for the second round PCR. Nested PCR increases the sensitivity and specificity of both DNA and RNA amplification.

Nested PCR was performed using *16S rDNA* universal eubacterial primers to screen for the bacterial species in the root canal samples.

Detection of *Enterococcus faecalis* was exclusively done by Multiplex PCR using three pairs of primers. The PCR reaction mixture of 25 µl volume consisted of 1 unit of *Taq* DNA polymerase (Bangalore genei, India.), 5 µl of 10X PCR buffer, three pairs of primers each of 0.5 µM of each primer (three) (Sigma-Aldrich Pvt Ltd, India), 0.2 mM of each dNTP (Medox Biotech India Pvt Ltd, India) and 5µl of DNA template.

The PCR products were loaded in to the 1.5% agarose gel and electrophoresed for 1- 1.5 hours in 0.5 X TBE buffer. After staining the gel with ethidium bromide solution, the DNA bands were visualized under UV light illumination (GELDOC). 100 bp DNA ladder (MEDOX) was used as a size marker and sterile milli Q water was used as blank control.

The amplicon size of first round PCR was 766bp and the second round PCR was 470bp. The second round product was further sequenced. All the *16S-rDNA* sequences obtained were blasted in the Genbank database. In addition, all *16S-rDNA* sequences were compared with the database sequences of the Ribosomal Database Project and the Human Oral Microbiome Database

All the clinical samples that were subjected to PCR analysis showed positive for *16S rDNA* Universal primer confirming the presence of bacteria in all the tested samples. The present study investigated the microbiological profile of 20 clinical samples obtained from Group 1 cases using Nested PCR. A total of 46 bacterial isolates belonging to 15 different microbial genera were identified which clearly shows the diversity in the bacterial population. A minimum of 2 microbial genera was identified in each root canal that was sampled. Out of 15 microbial genera that was identified in group 1 clinical samples, 7 microbial genera belonged to gram positive bacteria and 8 microbial genera belonged to gram negative bacteria constituting about 46.67% and 53.33 % of the total genera identified respectively.

Among 15 bacterial genera that was identified 12 were anaerobic and the remaining 3 were aerobic bacteria constituting about 80% and 20% of the total bacterial isolates identified respectively. These findings were in accordance with the previous studies done by Sundquist et al (1998) and Molander et al (1998).[17]

The total bacterial genera that were isolated from group 1 clinical samples can be broadly categorized in to 6 phyla namely Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Spirochaetes and Fusobacteria. Majority of the genera belonged to Firmicutes, followed by Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria and Spirochaetes.

The bacterial genera that was comparatively found in higher numbers in the present study in group 1 clinical samples were *Bacteroidetes* (6/20 cases), *Actinomyces* (6/20 cases) and *Enterobacter* (6/20 cases) each constituting about 30% of the total bacterial genera identified. *Prevotella* was isolated in 5/20 cases constituting to 25%, *Porphyromonas* in 4/20 cases constituting to 20%, *Actinobacter, Lactobacillus* and *Peptostreptococcus* were each isolated in 3/20 cases

accounting to 15% each. *Fusobacterium* spp was isolated in 2/ 20 cases constituting to 10%. The uncommon bacteria isolated in the group1 cases of the present study were *Lysinibacillus fusiformis* and *Microbacterium* spp each found in 2/ 20 cases constituting to about 10%.

Whereas, *Campylobacter* spp, *Streptococcus* spp *and Treponema denticola* were each found only in 1/20 cases sampled constituting the least percentage. Out of 20 samples in group1 that were investigated, *Enterococcus faecalis* was found only in 1 case (5%).

It was very evident from the present study that anaerobic bacteria were found in greater numbers than aerobic bacteria that were isolated from group 1 cases. This might be due to the fact that the low oxygen tension was conductive for the establishment of anaerobic bacteria. Moreover, necrotized pulp helps in the growth of bacteria that makes use of proteins as their main nutritional resource which explains why these bacteria reported to be the common members of the microbiota pertaining to this kind of environment.

The Firmicutes namely the *Lactobacillus, Peptostreptococcus, Lysinibacillus, Streptococcus* and *Enterococcus* comprised the majority

of the phyla isolated in the present study. This was is accordance with the previous culture and culture independent studies done by Sakamato et al, Munson et al, Gomes et al and Siquera et al.[11,18,30,36]

The uncommon bacteria namely the *Acinetobacter baumanii* belonging to the phyla Proteobacteria was isolated in 3 clinical samples investigated. The other uncommon bacteria that was isolated in this study was *Lysinibacillus fusiformis* which was identified in 2 cases. *Treponema denticola* belonging to the phyla spirochaete was found in only one case. Spirochaetes are abundantly present in subgingival samples of subjects having periodontitis. However, the selected subjects in the present study were free from periodontitis suggesting the absence of cross contamination from periodontal pockets. Therefore this low detection rate might indicate that this phylum may be not well adapted to the endodontic environment.

The present study also investigated the microbiological profile of 20 cases selected from secondary endodontic infection (group 2) using Nested PCR analysis. A total of 41 bacterial isolates were identified belonging to 17 different microbial genera. These findings suggest that

the bacterial diversity is not only seen in primary endodontic infection but also in secondary endodontic infection which can be greater than that it is known to date. This was in accordance with the study by Sakamota SM et al (2008) who used molecular analysis to isolate root canal microbiota associated with endodontic treatment failure.

Among the 17 different bacterial genera that was identified in group 2 clinical samples, 9 microbial genera belonged to gram positive bacteria and 7 belonged to gram negative bacteria constituting to 52.94% and 41.17% of the total bacterial genera isolated respectively. *Eubacterium* spp which was identified in one case can be considered as either gram positive or gram negative bacteria.

Out of 17 bacterial genera that were identified, 15 genera were anaerobic and only 2 were aerobic constituting about 88.23% and 11.76% of the total genera isolated respectively. The findings from the present study also showed that facultative anaerobes were the predominant ones among the anaerobes isolated. This was in accordance with the findings reported by Engstrom et al and Moller et al. This might be due to the fact that, facultative anaerobes are capable of being in a

quiescent phase showing low metabolic activity. The growth of these bacteria can be triggered by the changes in the nutritional conditions which in most of the cases might be through the coronal leakage.

The 17 bacterial genera that were isolated from group 2 cases can be categorized under 5 phyla namely the Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria and Fusobacteria. Firmicutes was the phyla found to comprise the majority of the bacterial genera that was identified in the present study. The bacterial genera that was comparatively found higher in the group 2 samples was *Streptococcus* spp which was identified in 6/20 cases among which *Streptococcus mitis* was identified in 2 samples, *Streptococcus sanguis* and *Streptococcus angiosus* were identified in 1 sample each. *Actinomyces* spp was isolated in 5/20 cases, *Prevotella* and *Fusobacterium* were isolated in 4/20 cases each. *Porphyromonas, Lactobacillus, Enterobacter, Bacillus subtilis, Propionibacterium and Bifidobacterium* were each seen in 2/20 cases investigated. *Eubacterium* spp, *Escherichia coli, Campylobacter* spp and *Clostridium* spp were seen in 1/20 cases each. The uncommonly isolated

bacterial species, *Butyvibrio* spp and *Veilonella* spp were each isolated in one case.

However, as far as secondary endodontic infections are concerned, there are numerous studies till date reporting *Enterococcus faecalis* to be the most predominant microorganism, showing prevalence from 38% to 81% (Sundquist et al 1998, Hancock et al 2001, Peciulline et al 2001, Pinheiro et al 2001, 2003, Sequira et al 2004, Zoletti et al 2006). Thus in this present study, *Enterococcus faecalis* was exclusively identified by Multiplex PCR using three set of primers as suggested by Ali Mahmoudpour et al (2007)[15].

The findings from the present study showed out of 20 clinical samples from group 2 that was investigated, *Enterococcus faecalis* was seen only in 4 cases constituting to a very less percentage (20%) in comparison with other studies. This finding is however in accordance with very few studies. (Rolph et al 2001, Sakamato et al 2008, Cheung et al 2001). This lower percentage of incidence might be due to the geographic location or inter individual differences or due to the nutritional differences. This finding regarding *Enterococcus faecalis* in

the present study argues the fact that there is slight over estimation of this species among the majority of the endodontists.

The other interesting finding in the present study which was noteworthy to be mentioned was the identification of *Lysinibacillus fusiformis* in 2 cases of primary endodontic infection. The entire Pubmed data base was searched with multiple keywords and was found that this is the first study to report this organism.

However, in the present study demographic and socioeconomic factors were not taken in to consideration, but the influence of these factors in the endodontic microbiota warrants further elucidation with a larger data set. Moreover, with the concept of Biofilms we are advancing in to an era where secondary endodontic infections are being considered to be Biofilm associated disease (Anderson et al 2012).[1] Hence it becomes more interesting if attempts are made to investigate in this aspect and to examine how different species could synergize with each other.

It is evident from the present study that, the endodontic microbiota varies according to the geographic location, proving the

proposed hypothesis. The results from the present study are believed to bring a temporal change in the endodontic treatment strategies. However, future studies investigating the endodontic microbiota pertaining to Indian population with a larger data set can lead to promising conclusions that will enable us to tailor the treatment protocol and render quality endodontics.

Summary

SUMMARY

This study was done to identify the bacteria present in patients with primary and secondary endodontic infections referred to department of conservative dentistry and endodontics, ragas dental college and hospital using Nested and Multiplex PCR.

Root canal samples were collected from 40 patients categorized into 2 groups with each group containing 20 patients each. Group 1 consisting of patients diagnosed with primary endodontic infection and Group 2 consisting of patients diagnosed with secondary endodontic infection. DNA extraction from the collected samples was done using boiling and lyses method. The extracted DNA was stored at -20^0 C until PCR assay. Bacterial identification was done using Nested PCR. The identification of *Enterococcus faecalis* was exclusively done using Multiplex PCR using three set of primers.

The entire PCR products were loaded in 1.5% agarose gel and electrophoresed for 1- 1.5 hours in 0.5X TBE buffer along with a 100 bp

ladder. After staining the gel with ethidium bromide solution, the DNA bands were visualized under UV illumination (GELDOC).

The amplicon size of the first round PCR was 766 bp and the second round was 470 bp. The second round product was further sequenced. All the *16S r DNA* sequences obtained were blasted in the Gene Bank Database. In addition, all *16S r DNA* sequences were also compared with database sequence of the Ribosomal Database Project and the Human Oral Microbiome Database. The identified bacteria were tabulated.

Conclusion

CONCLUSION

Within the limitations of the present study, it can be concluded that,

1. There is variation in the endodontic microbiota according to the geographic location.
2. Diversity in endodontic microbiota is not only seen in primary endodontic infections but also in secondary endodontic infections to a greater extent.
3. Firmicutes are the major phyla found in both primary and secondary endodontic infections
4. *Enterococcus faecalis* was found in 20% (4 out of 20) cases diagnosed with secondary endodontic infection.
5. *Enterococcus faecalis* was present only in 5% (1 out of 20) cases diagnosed with primary endodontic infection.
6. The one species which was newly identified in this present study is Lysinibacillus fusiformis, found in 2 out of 20 cases investigated under primary endodontic infection.

Bibliography

BIBLIOGRAPHY

1. **Anderson AC, Helling E, Vespermann R**

   Comprehensive analysis of secondary dental root canal infection: A combination of culture and culture independent approaches reveals new insights.

   Plose one 2012. 10:1371

2. **Bamman LL, Esrela C**

   Microbiological aspects in endodontics

   Endodontic Science 2009 ; 1: (2) : 258- 281

3. **Baumgartner JC, Siqueira JF, Xia T**

   Geographical Difference in Bacteria detected in endodontic infections using PCR

   Journal of endodontics 2004, 30: 141- 144

4. **Brito LCN, Teles FR, Franca EC**

   Use of multiple displacement amplification and checkerboard DNA-DNA hybridization to examine the microbiota of endodontic infections.

   Journal of clinical Microbiology 2007 ; 45 (9) : 3039-3049

5. **Cheung GSP, Ho MWM**

   Microbialflora of root canal treated teeth associated with asymptomatic periapical radiolucent lesions.

Oral Microbiology Immunology 2001; 16: 332-337

6. **Dewhirst FE**

   The Human Oral Microbiome

   Journal of Bacteriology 2010; 192:5002-5017

7. **Figdor D, Davis JK, Sundquist G**

   Starvation, survival, growth and recovery of Enterococcus faecalis in human serum.

   Oral Microbiology Immunology 2003; 18: 234-239

8. **Fouad AF, Joseph Zerella, BarryJ**

   Molecular detection of Enterococcus faecalis in root canals of therapy resistant endodontic infections

   Oral Surgery Oral medicine Oral Pathology Oral Radiology endodontics 2005;112-118

9. **Fouad AF, Kum KY, Barry J**

   Molecular characterization for the presence of Eubaterium species in endodontic infection

   Oral Microbiology Immunology 2003; 18: 249- 255

10. **Fouad AF, Barry J, Caimano M**

    Identification of Bacteria associated with Endodontic infections.

    Journal of Clinical Microbiology 2002; 40 (9) : 3223-3231

11. Gomes BPFA, Pinheiro ET, Sousa ELT

Microbiological examination of infected dental root canals

Oral Microbiology Immunology 2004; 19:71-76

12. Gomes BPFA, Pinheiro ET, Sousa ELT

Enterococcus faecalis in dental root canals detected by culture and polymerase Chain Reaction analysis

Oral Surgery Oral Medicine Oral Pathology Oral Radiology Endodontics 2006; 102:247-253

13. Gomes BPFA, Montagner F, Jacinto RL

Polymerase chain reaction of Porphyromonas gingivalis, Treponema denticola, Tanerella forsythia in primary endodontic infection

Journal of Endodontics 2007; 33(9)1049-1052

14. Kakehashi S, Stanley HR, Fitzgerald RJ

The effects of surgical exposures of dental pulp in germ free and conventional laboratory rats

Oral Surgery Oral Pathology Oral medicine 1965; 20 :340-349

15. Mahmoudpour A, Rahimi S, Sina M

Identification of E.faecalis in necrotic root canals using multiplex PCR

Journal of Oral sciences 2007; 49(3) 221-227

16. Manski RJ, Magder LS

Demographic ad socioeconomic predictors of dental care utilization

Journal of American Dental Association 1998; 129: 195-200

17. Molander A, Reit C, Dahlen G

Microbiological status of root filled teeth with apical periodontitis

International Endodontic Journal 1998; 31:1-7

18. Munson MA, Pittford T, Chong B

Molecular and cultural analysis of the microflora associated with endodontic infection.

Journal of Dental Research 2002; 81:761-766

19. Nair PNR

Pathogenesis of apical periodontitis and causes of endodontic failure.

Critical review of Oral Biology and Medicine 2004; 15(6):348-381

20. Paster BJ, Oisen L, Aas JA

The breadth of bacterial diversity in the human periodontal pockets and other oral sites

Periodontal 2000 2006; 42:80-87

21. Peciulliene V, Balcioniene I, Harld ME

Isolation of Enterococcus faecalis in previously root filled canals in a Lithuanian population

Journal of Endodontics 2000; 26 (10)

22. Peciuliene V, Manelieve R, Balcikonyte E

Microorganisms in root canal infection – A Review

Stomatolgya Baltic Dental and maxillofacial journal 2008; 10: 4-9

23. Pienheiro ET, Gomes BPFA, Teixeira FB

Evaluation of root canal microorganisms isolated from teeth with endodontic failure and their antimicrobial susceptibility

Oral Microbiology and Immunology 2003; 18:100-103

24. Pienheiro ET, Gomes BPFA, Ferraz CCR

Microorganisms from canals of root filled teeth with periapical lesions

International Endodontic Journal 2003; 36: 1-11

25. Pienheiro ET, Gomes BPFA, Drucker DB

Phenotypic and Genotypic identification of enterococci isolated from canals of root filled teeth with periapical lesions

Oral Microbiology and Immunology 2006; 21: 137-144

26. Portenier I, Waltimo TMT, Haapasalo M

Enterococcus faecalis – the root canal survivor and 'star' in post treatment disease

Endodontic Topics 2003; 6: 135- 159

27. Ribeiro AC, Matarazzo F, Faveri M

Exploring bacterial diversity of endodontic microbiota by cloning and sequencing 16S rRNA

Journal of endodontics 2011; 37:922-926

28. Rocas IN, Siqueira JF jr, Santos KRN

Association of Enterococcus faecalis with different forms of periradicular diseases

Journal of Endodontics 2004: 30 (5): 315-320

29. Rolph HJ, Lennon A, Riggio MP

Molecular identification of microorganisms from endodontic infections

Journal of Clinical Microbiology 2001; 39 (9) 3282-3289

30. Sakamato M, Siqueira JF, Rocas IN

Bacterial reduction and persistence after endodontic treatment procedures

Oral Microbiology Immunology 2007, 22:19-23

31. Schizrmeister JF, Libenow AL, Braun G

Detection and eradication of microorganisms in root filled teeth associated with periradicular lesions. An invivo study.

Journal of Endodontics 2007;33:536-540

32. Sedgley CM, Lennan SL, Clewell DB

Prevalence, phenotype and genotype of oral Enterococci

Oral Microbiology Immunology 2004; 19:95-101

33. Sedgley CM, Lennan SL, Appel be OK

Survival of E.faecalis in root canals exvivo

International Endodontic Journal 2005; 38: 735-742

34. Sedgley CM, Nagel A, Reit C

Real time quantitative PCR and cultural analyses of E.faecalis in Root Canals

Journal of Endodontics 2006; 32: 173-177

35. Siqueira JF, Rocas IN, Oliveira JC

Molecular detection of black pigmented bacteria in infections of endodontic region.

Journal of Endodontics 2001; 27: 563-566

36. Siqueira JF, Rocas IN, Jose F

Polymerase Chain Reaction based analysis of microorganism associated with failed endodontic treatment

Oral Surgery Oral Medicine Oral Pathology Oral Radiology Endodontics 2004;97:85-94

37. Siqueira JF, Rocas IN, Flavio RF

Selected Endodontic Pathogens in the apical third of infected root canals. A molecular investigations

Journal of Endodontics 2004; 30:9

38. Siqueira JF, Rocas IN

Uncultivated phylotypes and newly named species associated with primary and persistent endodontic infection

Journal of Clinical Microbiology 2005; 43 (7): 3314-3319

39. Siqueira JF, Young jung, Rocas IN

Differences in prevalence of selected species in primary endodontic infection from two distinct geographical locations

Oral Surgery Oral Medicine Oral Pathology Oral Radiology Endodontics 2005;99: 641-647

40. Siqueira JF, Rocas IN

Exploiting molecular methods to explore endodontic infections Part 1 Current molecular technologies for microbiological diagnosis

Journal of Endodontics 2005;31:6

41. Siqueira JF, Rocas IN, Paiva SS

Cultivable bacteria in infected root canals as identified by 16S rRNA gene sequencing

Oral Microbilogy Immunology 2007; 22: 266- 271

42. Siren ER, Haapasallo MPP, Ranta K

Microbiological findings and clinical treatment procedures in endodontic cases selected for microbiological investigation

Internantional Endodontic Journal 1997: 30: 91-95

43. Stuart CH, Schwartz SA, Beeson T

E. faecalis: Its role in root canal treatment failure and current concepts in retreatment

Journal of Endodontics 2006; 32: 93-98

44. Subramanian K, Mickel AK

Molecular analysis of persistent periradicular lesions and root end ends reveal a diverse microbial profile

Journal of Endodontics 2009; 35; 950-957

45. Sunde PT, Olsen I, Gilberto JD

Microbiota of periapical lesions refractory to endodontic therapy

Journal of Endodontics 2002; 28: 4

46. Williams JM, Trope M, Caplan DJ

Detection and quantification of E.faecalis by real time PCR(qPCR), reverse transcription PCR(RT PCR) and cultivation during endodontic treatment

Journal of Endodontics 2006; 32 (8):715-721

47. Zoletti GO, Siqueira JF, Santos KRN

Identification of Enterococcus faecalis in root filled teeth with or without periradicular lesions by culture dependent and independent approaches

Journal of Endodontics 2006;32:8:772-726